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Directed Against Telomerase RNA

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13. ABSTRACT (<i>Maximum 200 Words</i>) In most normal cells DNA is lost from the ends of the chromosomes (telomeres) at each cell cycle. In rapidly growing tumor cells this eventually results in chromosome instability and cell death. To overcome this problem most cancer cells re-express the telomerase enzyme which prevents telomere erosion. To establish whether expressing telomerase is essential to maintain the malignant phenotype in these tumor cells, we have used an antisense oligonucleotide approach targeting the RNA component of the telomerase enzyme. The oligonucleotides used carry a 2-5A moiety which activates RNaseL to selectively destroy the target RNA. Using this system we have shown that targeting telomerase causes rapid cell death in several different breast cancer cell lines <i>in vitro</i> . This cell death is not seen when the cells are treated with control oligos carrying mismatches in the target sequence. Cell death is due to apoptosis. When tumors from breast cancer cell lines were induced subcutaneously in nude mice and then treated directly with the 2-5A antisense oligos, tumor growth was reduced by up to 50% of that seen in tumors treated with control oligos. It appears therefore that targeting telomerase in breast cancer cells may provide a novel approach for cancer therapy.				
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FOREWORD

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N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.



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INTRODUCTION.

Telomerase is the riboprotein enzyme complex which prevents the ends of chromosomes from shortening below a critical length in cancer cells. This enzyme is normally not expressed in the majority of human cells after an early point in embryonic development but is reactivated in the vast majority (95%) of highly malignant cancer cells. It is thought to be an essential requirement for the maintenance of cell viability in cancer cells which express it. We have investigated whether inactivating telomerase in cancer cells using an antisense oligonucleotide approach targeting the RNA component of the enzyme will result in cell death. The oligonucleotides used carry a 2-5A moiety attached to the antisense molecule. 2-5A activates endogenous RNaseL which is normally found as an inactive monomer in the cytoplasm in most cells. In the presence of 2-5A the monomer dimerizes and become a potent RNase. Thus, the antisense molecule targets a specific RNA and the recruitment of RNaseL then selectively degrades the target. The overall aim of the project, therefore, is to determine whether inactivating telomerase can be developed as a viable form of anti cancer therapy for breast tumors. The initial series of experiments are designed to establish the conditions of treatment which will produce effective cell killing.

BODY

To investigate the response of breast cancer cells to treatment with 2-5A anti-hTR we selected three different cell lines, MDA468, MCF7 and T47D. The TRAP assay clearly demonstrated strong telomerase activity in all of these cell lines. This activity could be heat inhibited by incubating the cell extracts for 10 minutes at 80°C. In contrast, telomerase activity was not detected in either fibroblast cell lines MRC5 and WI38, or primary cultures of human vesicular endothelial cells or human corneal epithelial cells. Since the 2-5A-antisense system depends on recruiting RNase L to the molecule targeted for degradation, we investigated the levels of RNaseL expression using western blotting. All breast cancer cell lines demonstrated the presence of cellular RNase L using a monoclonal anti-human RNase L antibody. Amplification of RNase L mRNA using RT-PCR also revealed high levels of gene expression in all breast cancer cells.

We have treated cell lines T47D, MCF7 and MDA468 with 2-5A-anti-telomerase oligonucleotides (spA4-anti-hTR) and the results are shown in figure 1. In all 3 cases rapid cell death is induced in vitro following only 4-7 days of treatment. When we treat the same cells with a control oligonucleotide which contains an active 2-5A, but which carries mismatches within the targeting oligonucleotide (spA4-anti-hTR(M6)), no significant cell death is seen. When we treat cells with a perfect match oligonucleotide which is attached to a defective 2-5A moiety (spA2-anti-hTR), there is no significant cell death either. These experiments demonstrate that both the correct antisense targeting molecule as well as an active 2-5 moiety are required for cell death. Although it has been difficult to formally prove that cell death results from selective targeting of telomerase it is assumed that, because the controls have no effect on cell growth, the toxicity of the test compound is due to the disruption of enzyme function. To demonstrate that the oligonucleotides actually enter the cells we constructed a fluorescence tagged oligonucleotide and transfected it into breast cancer cells. The results are shown in figure 2 which demonstrate that large intracellular concentrations of oligos are achieved although there is heterogeneity from cell to cell which may account for the differential response of cells during the 8 day treatment period.

Since the dose of oligonucleotides will clearly be important in the overall cellular response, we titrated the cellular toxicity of the test compound between a concentration range of 0.1-5 μM . All oligos were administered using a cationic liposome (lipofectamine) to facilitate entry of the molecules into the cell. These experiments demonstrated a peak response at approximately 0.5 μM . We then investigated the dose response of MCF7 cells in the range 0.1-0.5 μM . As shown in figure 2 significant cell death was only achieved using oligo concentrations of 0.5 μM . This clearly demonstrated that there is a lower, effective concentration that must be used to achieve cell death. For all further experiments therefore we standardized the treatment using 0.5 μM oligos at a ratio of 1:4, DNA:lipofectamine. Since the initial treatments were every 12 hours we next investigated whether reducing the frequency of treatment could still achieve the same toxicity. When cells were treated either every 24 or 48 hours the same results were obtained. Treating every 72 hours resulted in initial toxicity followed by recovery of the cells. We therefore standardized the frequency of treatment to every 24 hours for all subsequent experiments.

Using this standard treatment protocol we next investigated the mechanism of cell death. Early indications were that cells were dying through apoptosis rather than necrosis. Using the ApoTag apoptosis kit it was possible to demonstrate that, within 5 days of treatment, all cells were being committed to apoptosis when treated with the 2-5A-anti-telomerase (figure 3). In contrast, very little apoptosis was seen when cells were treated with either the mismatch control or the 2-5A defective control oligos. These results demonstrated that active cell death was being induced by targeting the RNA component of telomerase, further supporting a causal role for the oligo treatment in the observed toxicity.

We have clearly established that tumor cells can be killed by using 2-5A-antisense against telomerase RNA. The next question was whether normal cells which do not express telomerase are affected or not. It has not been possible to establish progressively growing cultures of normal breast epithelial cells. We have therefore addressed this question indirectly and investigated normal human fibroblasts, WI38 and MRC5, and vascular and corneal endothelial cells. In all these 3 cases, normal cells which do not express telomerase, were relatively unaffected by the treatment. This demonstrates that there is unlikely to be toxicity of normal cells following treatment with 2-5A antisense.

To investigate whether tumor cells responded to antisense treatment *in vivo*, breast cancer cells were subcutaneously inoculated into nude mice and, after 8 days, tumors from the MDA468 cells reached a mean volume of 29.6mm^3 , (SD=3.9); MCF7 tumors reached a mean volume of 32.4mm^3 , (SD=10.2), and the T47D tumors reached a mean volume of 44.8mm^3 , (SD=11.7). Six tumors were used for each treatment arm which involved daily injections of the appropriate oligos. Tumor volume was then measured at even time points during the 10 day treatment period. For MDA468, treated with SpA4-hTR, the average size of the tumors was reduced to a mean volume of 27.7mm^3 , (SD=13), whereas tumors treated with spA4-hTR(M6) grew to an average 40.8mm^3 , (SD=9.6). The p value from the T-test = < 0.18 which was not statistically significant. In contrast, the average size of the MCF7 tumors after the 10 day treatment with SpA4-hTR was 15.2mm^3 (SD=4.2), and for SpA4-hTR(M6) treated tumors was 36mm^3 , (SD=4). This difference is statistically significant, the p value from T-test being < 0.001 . The same was true for tumors from T47D cells where, after 10 days treatment with SpA4-anti-hTR the average size was 18.7mm^3 , (SD=2.5), whereas tumors treated with SpA4-hTR(M6) were 43.5mm^3 , (SD=12.6). This difference is also statistically significant, with a $p < 0.01$. Thus, for two cell lines

treatment with the spA4-anti-hTR oligo reduced tumor volume by approximately 50% whereas the mismatch control did not affect tumor size.

To establish whether the rapid induction of apoptosis is due to a by-stander effect due to some signal secreted by cells undergoing apoptosis in the cultures, we transferred the tissue culture medium from cells treated with spA4-anti-hTR treatment to untreated cells daily. In these experiments no effect was seen on the cells which had never been challenged by anti-hTR oligos. The morphology of the cells also showed no differences compared with cultures grown in fresh medium. It appears, therefore, that the rapid cell death seen in anti-hTR treated cultures is due to the induction of apoptosis within the individual cells.

KEY ACCOMPLISHMENTS

- Demonstration that targeting telomerase using 2-5-anti-telomerase oligonucleotides results in rapid cell death *in vitro* in a variety of different cell lines.
- Establishment of the effective treatment regimen in terms of dose, frequency of treatment and combination with liposomes.
- Exclusion of any significant bystander effect.
- Demonstration that normal cells are unaffected by the treatment.
- Demonstration that apoptosis is induced by targeting telomerase
- Demonstration that tumor growth *in vivo* can be reduced by direct treatment of the tumors with antisense against human telomerase.

REPORTABLE OUTCOMES

None

CONCLUSIONS

We have demonstrated that targeting telomerase with antisense oligonucleotides in breast cancer cells results in the rapid induction of apoptosis and cell death within 8-10 days of treatment. We have established the treatment regimen *in vitro* which involves administration of the antisense molecules every 24 hours, in the presence of lipofectamine at a concentration of 0.5 μ M. The induction of apoptosis occurs progressively throughout the treatment period. The apoptotic response is not due to a bystander effect but rather is specific to individual cells receiving the antisense molecules. When tumors are induced subcutaneously in nude mice and then treated daily by direct injection of antisense oligos, tumor growth is reduced by up to 50%. The effect of the 2-5A-anti-telomerase appears to be specific for the tumor cells since normal cells treated in a similar fashion do not undergo apoptosis and furthermore treatment with either mismatch oligos or ones defective in their ability to recruit RNase L does not result in significant cell death either. We feel that these preliminary experiments provide the proof of principle that disrupting telomerase function in tumor cells has the potential for a novel therapeutic approach. The next task will be to investigate the mechanism underlying the action of these 2-5A-antisense molecules and investigate whether they are stable enough to be used directly in the treatment of tumors.

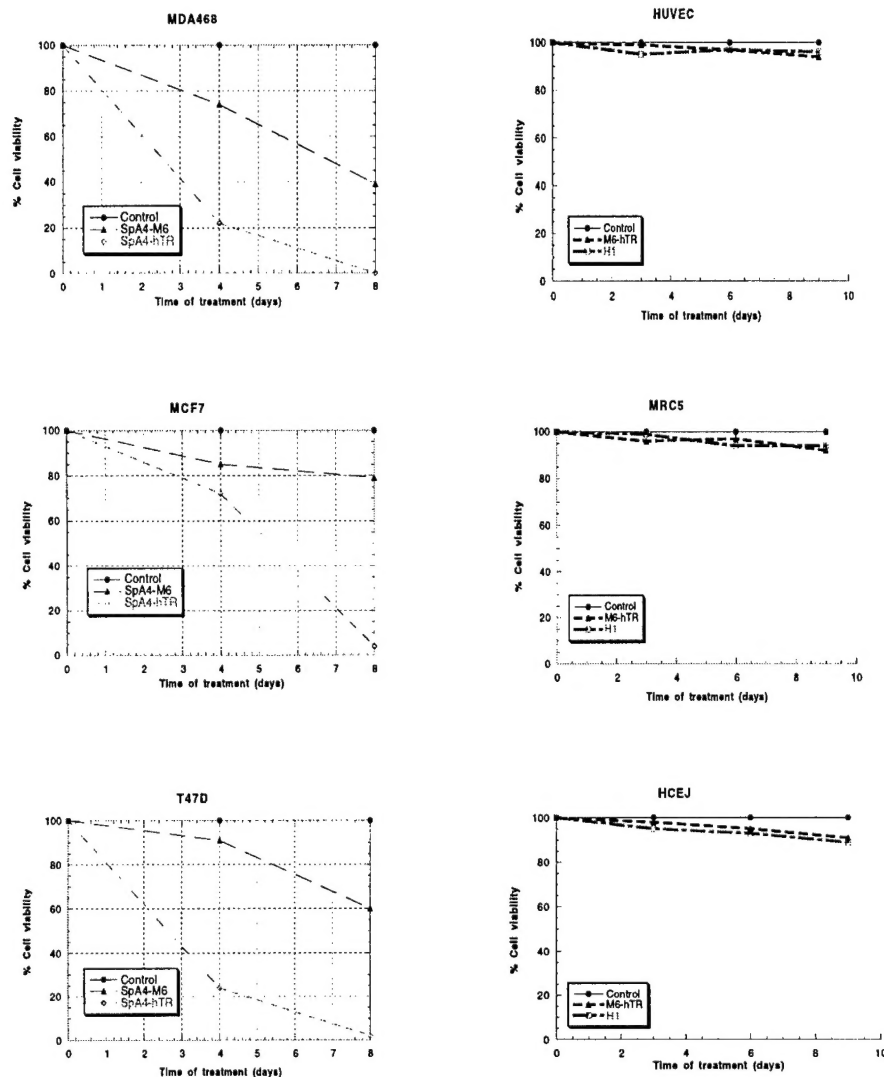


Figure 1: Dose response curves for 3 breast cancer cell lines (left) and normal human epithelial and fibroblast cultures (right). Cell viability is normalized against cells treated with lipofectamine alone (control). Breast cancer cell lines treated with the spA4-hTR show a rapid decline in cell viability over the 8 days period whereas treatment with the mismatch control (spA4-M6) show a much reduced loss of cell viability. In normal cells, neither oligonucleotide has any significant effect on cell viability.

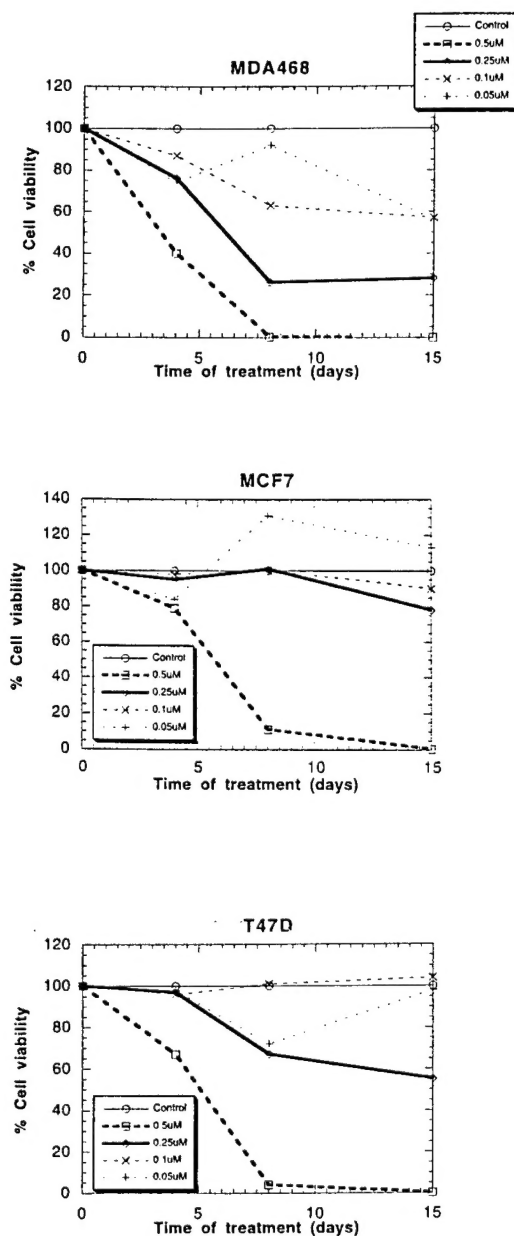


Figure 2: Effects of decreasing the overall concentration of oligonucleotides on cell viability in 3 different cell lines. Although the specific response for individual cell lines differs slightly, the overall result is that using concentration of less than 0.5 uM results in a reduced toxicity.

Flow Cytometric Analysis of Apoptosis in Breast Cancer MDA468 Cell Line

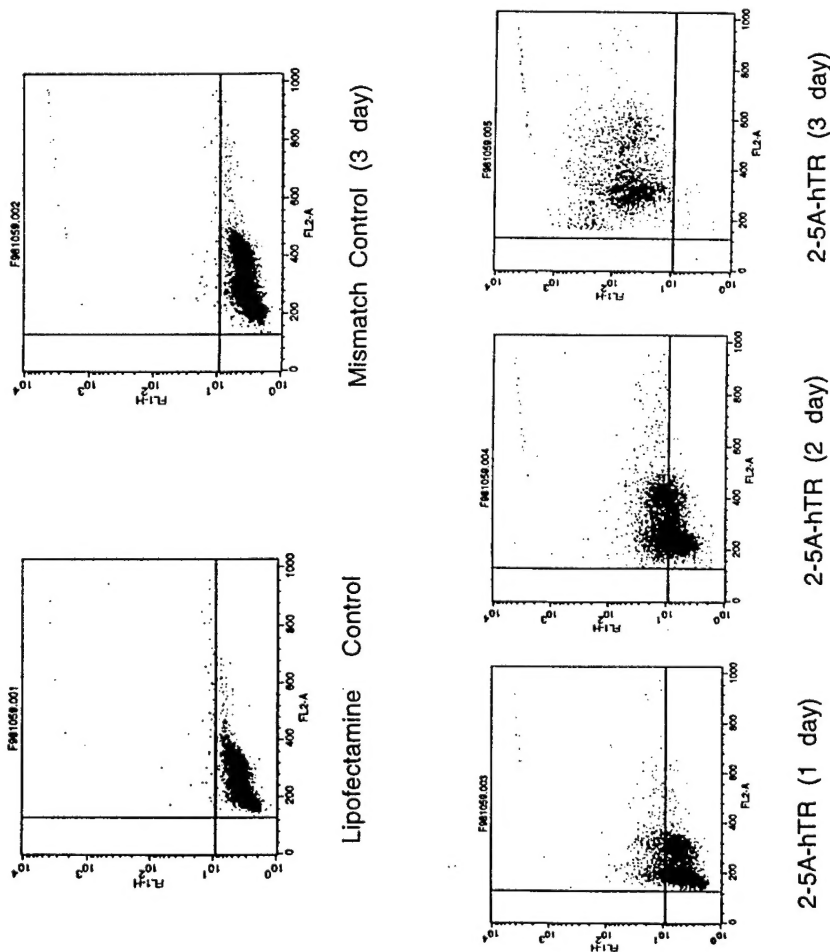


Figure 3: The ratio between intact and fragmented DNA in the cell population provides an accurate assessment of the extent of apoptosis. Using flow cytometry, apoptotic cells appear in the main window - normal cells stay below the horizontal line within the graph. These results show that cells treated with lipofectamine alone do not undergo any appreciable apoptosis during a 3 day period. Cells treated with the mismatch oligonucleotide do not demonstrate apoptosis either. In contrast, cells treated with the 2-5A-anti-hTR show increasing numbers of cells undergoing apoptosis on successive days until day 3 where the majority of cells are in apoptosis.